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APPLICANTS: Evans et al.

EXAMINER: Schnizer

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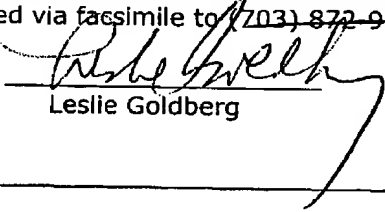
FILED: January 29, 2002

FOR: Method for Producing Circular or Multimeric Protein Species
in vivo or *in vitro* and Related Methods

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Leslie Goldberg

Sir:

DECLARATION UNDER 37 C.F.R. §1.131

As a below named inventor, I hereby declare that:

1. My name is Dr. Ming-Qun Xu, Senior Scientist at New England Biolabs Inc. My resume is attached. While Tom Evans and I worked together on the claimed invention, the attached notebook pages are obtained from my laboratory notebook.

2. The Examiner has rejected claims 12, 14 and 16 in the above application, directed to a method for the *in vivo* production of a cyclic polypeptide, citing Scott et al. *PNAS* 96, pp 13638-13643 which was submitted October 7, 1999 and published November 23, 1999. In support of our assertion that we conceived and diligently reduced to practice the claimed invention prior to November 23, 1999 and indeed prior to October 7, 1999, copies of three pages from the laboratory notebook of Ming-Qun Xu are attached with redaction of the dates. A discussion of each page is presented below. Applicants submitted a paper for publication on December 8, 1999 that was published March 31, 2000 (copy attached hereto). References to this paper are made in conjunction with the discussion of the earlier completed experimental data attached hereto.

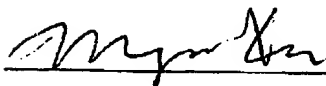
3. On the first page of the lab notebook attachment, a sequence for maltose-binding protein (MBP) is shown that was determined after Factor Xa cleavage. This sequence established that the MBP had been circularized by *in vivo* splicing. The sequence obtained corresponded to the C-terminal end of the protein (GTLEKFAEY) adjacent to the N-terminus (C here X, FNISTGM). The diagram on the left of the sequence depicts a circular molecule with the C-terminal end of MBP adjacent to the N-terminus. The third diagram on the page shows the N-terminal end of the MBP protein (XFNISTGM) that results when a single split intein is cleaved at the N-terminal end of the MBP protein. The first diagram on the page provides the sequence of the split intein (referring to Ssp DnaE intein) in the intein-MBP fusion protein, identified as MEB 16, in the absence of cleavage.

4. The second submitted page shows the sequence associated with two bands from a gel where the higher band is suspected of being circular MBP while the lower band is thought to be linear MBP following intein cleavage. Amino acid sequencing confirmed these assumptions. This data is also provided on page 9094 of the attached Evans reference, with the paragraph identified by (1).

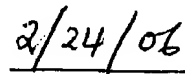
5. Page 181 of the lab notebook shows a cartoon at the top of the page describing the design of the experiment to test whether splicing of the split intein occurred and if so whether linear or circular MBP was produced from splicing of both split inteins from the ends of the MBP protein. The gel also present on the page shows that in fact circularized MBP is obtained as seen by a particular band on the gel (lane 6) and that the circular protein band disappears after cleavage with Factor Xa to produce a band corresponding to linear MBP (lane 7). This gel is reproduced in Figure 5A of the Evans reference (p. 9093) after the experiment was repeated. Lane 4 in Figure 5A corresponds to lane 6 on the gel on attached lab notebook page 181 (read from left to right) and lane 5 in Figure 5A corresponds to lane 7 of the gel of page 181.

6. The evidence provided above teaches for the first time the *in vivo* production of a cyclic polypeptide.

7. I further declare under penalty of perjury pursuant to laws of the United States of America the foregoing is true and correct.



Dr. Ming Xu



Date

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Education:**B.S.** 1982 University of Science and Technology of China**Ph.D.** 1989 Molecular Biology, Department of Biological Sciences, State University of New York at Albany**Work History**1990-1992: Postdoctoral research on self-splicing introns with Dr. David Shub, SUNY at Albany
Discovered the first eubacterial intron (Xu et al. Science, 1990)

1992 – 1994: Postdoctoral research with Dr. Fran Perler at New England Biolabs, Inc. Performed the first in vitro protein splicing experiment (Xu et al., Cell 1993)

1994 – 1997: Staff Scientist at New England Biolabs, Inc. Investigated the chemical mechanism of protein self-splicing. Developed the intein-based affinity purification system – IMPACT.

1997 – present: Senior Scientist at New England Biolabs, Inc. Structural and mechanistic studies of self-splicing inteins. Engineered inteins for protein semisynthesis, protein backbone cyclization and trans-splicing.

2001-2005: Managing Director, New England Biolabs (Beijing) Ltd.

Current Research Interest:

Structural and mechanistic studies of protein splicing have been conducted by collaboration to solve the crystal structures of self-splicing-inteins derived from the *dnaB* and *dnaE* genes of *Synechocystis* sp. PCC6803. The finding that the DnaE intein precursor structure contains a zinc ion, the only identified inhibitor of both *cis*- and *trans*-splicing, chelating the highly conserved Cys160 and Asp140 reveals the structural basis of Zn²⁺-mediated inhibition. These structural

studies provide insight into the sequential reaction property of protein splicing as well as the strategies to utilize inteins for protein engineering.

A number of intein engineering projects have been carried out for protein/antibody affinity purification, protein labeling and tagging, ligation and cyclization of expressed proteins. The Intein-mediated protein ligation (IPL) method has been applied to new fields including antibody characterization, epitope mapping, kinase/phosphatase assays for analysis via peptide arrays, western blots and ELISA.

PUBLICATIONS:

1. Ming-Qun Xu, Inca Ghosh, Samvel Kochinyan and Luo Sun. Intein-mediated Peptide Arrays for Epitope Mapping and Kinase/Phosphatase Assays. *Methods in Molecular Biology*, vol., *Microarrays: Methods and Protocols* Edited by J.B. Rampal. Humana Press Inc., Totowa, NY. In press.
2. Sun Ping, Sheng Ye, Sebastien Ferrandon, Evans, T.C. Jr., Ming-Qun Xu, Zihe Rao (2005) Crystal structures of an intein from the split *dnalE* gene of *Synechocystis* sp. PCC6803 reveal the catalytic model of intein without the penultimate histidine and the mechanism of zinc ion inhibition of protein splicing. *J. Mol. Biol.* 353: 1093-1105.
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